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<p>(54) Title: METHODS AND COMPOSITIONS FOR INHIBITING THE FUNCTION OF POLYNUCLEOTIDE SEQUENCES</p> <p>(57) Abstract</p> <p>A therapeutic composition for inhibiting the function of a target polynucleotide sequence in a mammalian cell includes an agent that provides to a mammalian cell an at least partially double-stranded RNA molecule comprising a polynucleotide sequence of at least about 200 nucleotides in length, said polynucleotide sequence being substantially homologous to a target polynucleotide sequence. This RNA molecule desirably does not produce a functional protein. The agents useful in the composition can be RNA molecules made by enzymatic synthetic methods or chemical synthetic methods <i>in vitro</i>; or made in recombinant cultures of microorganisms and isolated therefrom, or alternatively, can be capable of generating the desired RNA molecule <i>in vivo</i> after delivery to the mammalian cell. In methods of treatment of prophylaxis of virus infections, other pathogenic infections or certain cancers, these compositions are administered in amounts effective to reduce or inhibit the function of the target polynucleotide sequence, which can be of pathogenic origin or produced in response to a tumor or other cancer, among other sources.</p>			
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METHODS AND COMPOSITIONS FOR INHIBITING THE FUNCTION OF POLYNUCLEOTIDE SEQUENCES

Field of the Invention

The present invention relates to polynucleotide compositions which have an inhibitory or other regulatory effect upon the function of certain target polynucleotide sequences present in a mammalian cell, and for methods of using the compositions in therapeutic, prophylactic, diagnostic and research methods.

Background of the Invention

Polynucleotide compositions have been described for pharmaceutical uses, primarily for treatment or prophylaxis of disease in mammals, as well as in research in such fields. Specifically a great deal of activity presently surrounds the use of polynucleotide compositions in the treatment of pathogenic extracellular and intracellular infections, such as viral, bacterial, fungal infections, and the like. As one example, DNA vaccines are described to deliver to a mammalian cell *in vivo* an agent which combats a pathogen by harnessing the mammalian immune system. Thus, such vaccines are designed to express, for example, a viral protein or polypeptide, and elicit a humoral or cellular immune response upon challenge by the infective agent. Gene therapy vectors, on the other hand, are polynucleotide compositions generally designed to deliver to a mammalian cell a protein which is either not expressed, expressed improperly or underexpressed in a mammal. Such vectors frequently must address species specific immune responses to the those polynucleotide sequences that are recognized as antigenic or which evoke an unwanted cellular immune response.

Still other therapeutic uses of polynucleotide compositions are for the delivery of missing or underexpressed proteins to a diseased mammalian patient. Furthermore, polynucleotides are useful themselves as *in vivo* reagents, in diagnostic/imaging protocols, as reagents in gene therapy, in antisense protocols and in vaccine applications or otherwise as pharmaceuticals used to treat or prevent a variety of ailments such as genetic defects, infectious diseases, cancer, and autoimmune diseases.

Polynucleotides are also useful as *in vitro* reagents in assays such as biological research assays, medical, diagnostic and screening assays and contamination detection assays.

A host of problems well-known to the art has prevented the numerous 5 polynucleotide compositions from becoming widely accepted as useful pharmaceuticals. Thus, there are few such DNA vaccines or therapeutics which have yet been accepted by the medical community for the treatment of disease in mammals.

Phenomena have been observed in plants and nematodes that are mediated by 10 polynucleotide compositions, and are referred to as post-transcriptional gene silencing and transcriptional silencing. This phenomenon demonstrates that transfection or 15 infection of a plant, nematode or *Drosophila* with a virus, viroid, plasmid or RNA expressing a polynucleotide sequence having some homology to a regulatory element, such as a promoter or a native gene or a portion thereof already expressed in that cell, can result in the permanent inhibition of expression of both the endogenous regulatory 20 element or gene and the exogenous sequence. This silencing effect was shown to be gene specific. See, for example, L. Timmons and A. Fire, *Nature*, 395:354 (Oct. 29, 1998); A. Fire *et al*, *Nature*, 391:806-810 (Feb. 19, 1998); and R. Jorgensen *et al*, *Science*, 279:1486-1487 (March 6, 1998)]. A DNA plasmid encoding a full-length pro-alpha 1 collagen gene was transiently transfected into a rodent fibroblast tissue 25 cell line and a "silencing" effect on the native collagen gene and the transiently expressed gene observed [Bahramian and Zarbl, *Mol. Cell. Biol.*, 19(1):274-283 (Jan. 1999)].

See, also, International Patent Application No. WO98/05770, published 25 February 12, 1998, which relates to gene inhibition by use of an antisense RNA with secondary structures, and/or in combination with double stranded RNase.

International Patent Application No. WO99/53050, published October 21, 1999, also relates to reducing phenotypic expression of a nucleic acid, particularly in plant cells, by introducing chimeric genes encoding sense and anti-sense RNA molecules.

There exists a need in the art for polynucleotide compositions and methods of 30 using same to inhibit the function of polynucleotide sequences which are disease-

causing in mammals, such as polynucleotide sequences essential for the replication of viruses and other intracellular pathogens in mammalian cells, or sequences of extracellular mammalian pathogens, or sequences of tumor antigens which mediate the spread of cancer in a mammal, and the like, without adversely affecting essential gene 5 sequences in the mammal.

Summary of the Invention

In one aspect, the invention provides a composition for inhibiting the function of a target polynucleotide sequence in a mammalian cell. The composition comprises an agent that provides to a mammalian cell an at least partially double-stranded RNA 10 molecule comprising a polynucleotide sequence of at least about 200 nucleotides in length. The polynucleotide sequence is substantially homologous to the target polynucleotide sequence, which can be a polynucleotide sequence, e.g., of a virus or other intracellular pathogen, a polynucleotide sequence of a cancer antigen or of an essential tumorigenic regulatory sequence, a polynucleotide sequence of an 15 extracellular pathogen present in a mammal, or any other polynucleotide sequence which is desired to be "turned off" in a cell. This RNA molecule preferably does not produce a functional protein. This RNA molecule is preferably substantially non-homologous to naturally-occurring, essential mammalian polynucleotide sequences. In one embodiment, the agent of this composition is an RNA molecule made by 20 enzymatic synthetic methods or chemical synthetic methods *in vitro*. In another embodiment, the RNA molecule may be generated in a recombinant culture, e.g., bacterial cells, isolated therefrom, and used in the methods discussed below. In another embodiment the agent of this composition generates the RNA molecule *in vivo* after delivery to the mammalian cell.

25 In another aspect, the invention provides a pharmaceutical composition comprising one or more of the compositions described immediately above and specifically hereinbelow, and an optional second agent that facilitates polynucleotide uptake in a cell, in a pharmaceutically acceptable carrier. Such compositions are useful for treating intracellular pathogenic infections, such as viruses. Other such

compositions are useful for treating certain cancers. Other such compositions are useful for treating certain extracellular pathogens. Still other such compositions are useful for treating any disease or disorder wherein inhibiting the function of a polynucleotide sequence in a mammal is desirable for therapy or vaccine use.

5 In still another aspect, the invention provides a method for treating a viral infection in a mammal by administering to the mammal one or more of the above-described compositions wherein the target polynucleotide is a virus polynucleotide sequence necessary for replication and/or pathogenesis of the virus in an infected mammalian cell, along with an optional second agent that facilitates polynucleotide 10 uptake in a cell, in a pharmaceutically acceptable carrier. This composition is administered in an amount effective to reduce or inhibit the function of the viral sequence in the cells of the mammal.

In yet a further aspect, the invention provides a method for preventing a viral infection in a mammal by administering to the mammal one or more of the above-15 described compositions wherein the target polynucleotide is a virus polynucleotide sequence necessary for replication and/or pathogenesis of the virus in an infected mammalian cell, with an optional second agent that facilitates polynucleotide uptake in a cell, in a pharmaceutically acceptable carrier. This composition is administered in an amount effective to reduce or inhibit the function of the viral sequence upon 20 subsequent introduction of the virus into the mammalian cells.

In still another aspect, the invention provides a method for treatment or prophylaxis of a virally induced cancer in a mammal by administering to the mammal one or more of the above described compositions in which the target polynucleotide is a sequence encoding a tumor antigen or functional fragment thereof or a regulatory 25 sequence, which sequence function is required for the maintenance of the tumor in the mammal. The compositions can contain an optional second agent that facilitates polynucleotide uptake in a cell, and a pharmaceutically acceptable carrier. The composition is administered in an amount effective to reduce or inhibit the function of the tumor-maintaining sequence in the mammal.

In another aspect, the invention provides a method for the treatment or prophylaxis of infection of a mammal by an intracellular pathogen. The mammal is administered one or more of the compositions herein described wherein the target polynucleotide is a polynucleotide sequence of the intracellular pathogen necessary for 5 replication and/or pathogenesis of the pathogen in an infected mammalian cell. The composition is administered with an optional second agent that facilitates polynucleotide uptake in a cell, in a pharmaceutically acceptable carrier, in an amount effective to reduce or inhibit the function of the sequence in the mammal.

In another aspect, the invention provides a method for the treatment or 10 prophylaxis of infection of a mammal by an extracellular mammalian pathogen. The mammal is administered one or more of the compositions herein described wherein the target polynucleotide is a polynucleotide sequence of the extracellular pathogen necessary for replication and/or pathogenesis of the pathogen in an infected mammal. The composition is administered in a pharmaceutically acceptable carrier, in an 15 amount effective to reduce or inhibit the function of the sequence in the mammal. It may be administered with an optional second agent that facilitates polynucleotide uptake by the pathogenic cell.

In still another aspect, the invention provides a method of treatment or prophylaxis of cancer in a mammal. The mammal is administered one or more of the 20 above-described compositions, wherein the target polynucleotide is a polynucleotide sequence of an abnormal cancer-causing gene or non-expressed regulatory sequence in a mammal, which also possesses a normal copy of the gene or regulatory sequence. According to this aspect, the differences between the abnormal sequence and the 25 normal sequence are differences in polynucleotides. The composition is administered with an optional second agent that facilitates polynucleotide uptake in a cell, in a pharmaceutically acceptable carrier, and in an amount effective to reduce or inhibit the function of the abnormal sequence in the mammal.

In yet a further aspect, the invention involves a method for treating a disease or disorder in a mammal comprising administering to the mammal having a disease or 30 disorder characterized by expression of polynucleotide product not found in a healthy

nucleotides with a homology in that window of at least 90% to a similar 30 nts region of the target sequence.

In yet another embodiment, the RNA polynucleotide sequence desirably has an overall homology of at least 10% to the target sequence and contains at least two 5 windows of 30 contiguous nucleotides with a homology in the windows of at least 50% to similar 30 nts regions of the target sequence. Other embodiments of this formula can be developed by one of skill in the art.

In a second preferred embodiment, the RNA polynucleotide sequence desirably has an overall homology of at least 10% to the target sequence and contains 10 at least one segment (window) of 5 contiguous nucleotides with absolute homology in that window to a 5 nts region of the target sequence, using the MACVECTOR program with a default annealing temperature of 37°C. In another variant of this embodiment, the RNA polynucleotide sequence desirably has an overall homology of at least 30% to the target sequence and contains at least one window of 5 contiguous 15 nucleotides with absolute homology to a 5 nts region of the target sequence. In another embodiment, the RNA polynucleotide sequence desirably has an overall homology of at least 50% to the target sequence and contains the above described 5 nt absolutely homologous window. Other variants of this embodiment can be developed by one of skill in the art.

20 The presence of the windows referred to in the formulae above permits the overall homology of the remainder of the sequence to be low; however it is anticipated that a low overall homology is likely to affect the dosage of the therapeutic compositions described below adversely. An increase in the number of such windows in the RNA polynucleotide sequence is likely to permit the overall homology of the 25 rest of the sequence to be low, but not affect the dosage

It should be understood that selection of the necessary homology, selection of the defaults for the program and selection of the program employed to calculate homology is within the skill of the art, given the teachings of this specification and the knowledge extant in the scientific literature.

The RNA molecule polynucleotide sequence is also desirably substantially non-homologous to any naturally occurring, normally functioning, and essential mammalian polynucleotide sequence, so that the RNA molecule polynucleotide sequence does not adversely affect the function of any essential naturally occurring 5 mammalian polynucleotide sequence, when used in the methods of this invention. Such naturally occurring functional mammalian polynucleotide sequences include mammalian sequences that encode desired proteins, as well as mammalian sequences that are non-coding, but that provide for essential regulatory sequences in a healthy mammal. Essentially, the RNA molecule useful in this invention must be sufficiently 10 distinct in sequence from any mammalian polynucleotide sequence for which the function is intended to be undisturbed after any of the methods of this invention is performed. As described for determining the homology to the target sequence above, one of skill in the art may have resort to the above-identified computer algorithms to define the essential lack of homology between the RNA molecule polynucleotide 15 sequence and the normal mammalian sequences. Thus, in one exemplary embodiment, the homology between the RNA polynucleotide and the selected normal sequence is less than the homologies of the formulae described above. More preferably, there is almost no homology at all between the RNA polynucleotide and any normal mammalian sequence. It should be understood that selection of the necessary 20 homology is within the skill of the art, given the teachings of this specification and the knowledge extant in the scientific literature.

Finally, yet another desirable attribute of the RNA molecule of the composition of the present invention is that it does not produce a functional protein, or alternatively, is not translated. The RNA molecule or the delivery agent can be 25 engineered in a variety of known ways, so as to optionally not express a functional protein or to optionally not interact with cellular factors involved in translation. Thus, for example, the agent, whether it be a synthesized RNA molecule or an agent which becomes an RNA molecule *in vivo*, lacks a poly-adenylation sequence. Similarly, the agent can lack a Kozak region necessary for protein translation. In another 30 embodiment, the RNA molecule can also lack the native initiating methionine codon.

In still another embodiment, the RNA molecule polynucleotide sequence lacks a cap structure. In yet a further embodiment, the RNA molecule has no signals for protein synthesis. In still another embodiment, the RNA molecule contains no coding sequence or a functionally inoperative coding sequence. In still another embodiment, 5 the RNA sequence can be punctuated with intronic sequences. In yet a further embodiment, a hairpin sequence can be placed before the native initiation codon, if present. In still another embodiment, the RNA molecule can be an RNA/DNA hybrid as described above. All such embodiments can be designed by resort to the known teachings of, e.g., such texts as cited below.

10 The following are various specific embodiments that may be used to achieve polynucleotide inhibition as described herein. It should be recognized that the various RNA (and RNA/DNA hybrid) structures described below may be used singly or in any combination of two or more, e.g., a lariat (sense or antisense) and/or a complementary circular and/or linear molecule. The antisense lariat or circle structures may also be 15 used alone. Furthermore, these structures may include regions of self complementarity (e.g., tandem sense and antisense sequences) as well as additional antisense sequences relative to a desired target. Throughout this document the term "antisense" is used to mean complementary to and capable of hybridizing with any mRNA.

20 In one embodiment, polynucleotides in the form of "lariats" may be utilized. Lariats contain a 2'-5' phosphodiester linkage as opposed to the usual 3'-5' linkage. Such structures are formed in splicing reactions catalyzed by spliceosomes and self-cleaving ribozymes. These structures are either intermediates or by-products of 25 splicing reactions. They can be prepared *in vivo* through expression (transcription) in a cell or prepared *in vitro*. Lariats form when a free 5' phosphoryl group of either a ribose or deoxyribose becomes linked to the 2' -OH of a ribose in a loop back fashion. The lariats may contain 10 or more nucleotides in the loop or may be a complete 30 circle, with the loop back linkage in each case being 2'-5'. A lariat linking the terminal nucleotides produces a circle-like structure. The loops and/or the stem can contain either the sense and the antisense sequences in tandem in a single molecule, or

each single lariat contains either a sense or an antisense sequence. The lariats that contain sense and antisense in separate molecules may be administered together as a double-stranded form or the antisense lariat may be used singly to form a double strand with the mRNA in the cell. Lariats may be RNA or a DNA hybrid, with the 2'-5' linkage effected through the 2' -OH of the RNA portion of the hybrid [Rees C and Song Q. Nucl. Acid Res., 27, 2672-2681 (1999); Dame E *et al*, Biochemistry, 38, 3157-3167, 1999; Clement J.Q. *et al*, RNA, 5, 206-220, 1999; Block T and Hill J. J. Neurovirol., 3, 313-321, 1997; Schindewolf CA and Domdey H., Nucl. Acid Res., 23, 1133-1139 (1995)].

10 In another embodiment, a circular RNA (or circular RNA-DNA hybrid) can be generated through a 2'-5' or a 3'-5' linkage of the terminii. These may be generated enzymatically through RNA ligase reactions using a splinter to bring the ends in proximity *in vitro*, or through the use of self splicing ribozymes (*in vivo* and *in vitro*). The desired inhibition may be achieved by providing one or more RNA circles, made *in vitro* or expressed *in vivo*, including single circles with or without self complementarity, as well as double stranded circular RNA (both sense and antisense strands relative to the target polynucleotide), or two circles of single-stranded RNA which have regions of complementarity to each other as well as one having complementarity to a target.

15 Another embodiment utilizes single RNA (or RNA-DNA hybrid) antisense circles (circular RNA without self complementarity which is complementary to the target mRNA). Still another embodiment utilizes RNA-DNA circles or a circular DNA molecule complementary to a target mRNA molecule. Single circles with tandem sense and antisense sequences (in any order) which have complementarity to a target message may be used as the composition which inhibits the function of the target sequence. It may be preferred to use circular molecules having such self-complementary sequences which may form rod-like sections, as well as additional antisense sequences to the target [Schindewolf CA and Domdey H. Nucl. Acid Res., 23, 1133-1139 (1995); Rees C and Song Q., Nucl. Acid Res., 27, 2672-2681 (1999);

20 Block T and Hill J., J. Neurovirol., 3, 313-321(1997)].

In yet a further embodiment, the composition which inhibits the target sequence is a capped linear RNA. Whether the dsRNA is formed *in vitro* or *in vivo*, either one or both strands may be capped. In circumstances where cytoplasmic expression would not ordinarily result in capping of the RNA molecule, capping may 5 be accomplished by various means including use of a capping enzyme, such as a vaccinia capping enzyme or an alphavirus capping enzyme. A capped antisense molecule may be used to achieve the desired post transcriptional silencing of the target gene. Capped RNA may be prepared *in vitro* or *in vivo*. RNA made in the nucleus by RNA polII ordinarily is capped. Cytoplasmically expressed RNA may or 10 may not be capped. Capping can be achieved by expressing capping enzymes of cytoplasmic viruses. Either both capped or one capped and one uncapped or both uncapped RNA or RNA-DNA hybrid sequences may be used in these compositions. Capped or uncapped antisense molecule may be used, singly or in any combination with polynucleotide structures described herein.

15 The RNA molecule according to this invention may be delivered to the mammalian or extracellular pathogen present in the mammalian cell in the composition as an RNA molecule or partially double stranded RNA sequence, or RNA/DNA hybrid, which was made *in vitro* by conventional enzymatic synthetic methods using, for example, the bacteriophage T7, T3 or SP6 RNA polymerases 20 according to the conventional methods described by such texts as the Promega Protocols and Applications Guide, (3rd ed. 1996), eds. Doyle, ISBN No. 1-882274-57-1.

Alternatively these molecules may be made by chemical synthetic methods *in vitro* [see, e.g., Q. Xu *et al*, Nucl. Acids Res., 24(18):3643-4 (Sept. 1996); N. 25 Naryshkin *et al*, Bioorg. Khim., 22(9):691-8 (Sept. 1996); J. A. Grasby *et al*, Nucl. Acids Res., 21(19):4444-50 (Sept 1993); C. Chaix *et al*, Nucl. Acids Res., 17(18):7381-93 (1989); S.H. Chou *et al*, Biochem., 28(6):2422-35 (Mar. 1989); O. Odai *et al*, Nucl. Acids Symp. Ser., 21:105-6 (1989); N.A. Naryshkin *et al*, Bioorg. Khim., 22(9):691-8 (Sept. 1996); S. Sun *et al*, RNA, 3(11):1352-1363 (Nov. 1997);

and 65,643,771, incorporated herein by reference. Microorganisms useful in preparing these delivery agents include those listed in the above cited reference, including, without limitation, *Escherichia coli*, *Bacillus subtilis*, *Salmonella typhimurium*, and various species of *Pseudomonas*, *Streptomyces*, and

5 *Staphylococcus*.

Still other delivery agents for providing the information necessary for formation of the desired, above-described RNA molecules in the mammalian cell include live, attenuated or killed, inactivated viruses, and particularly recombinant viruses carrying the required RNA polynucleotide sequence discussed above. Such

10 viruses may be designed similarly to recombinant viruses presently used to deliver genes to cells for gene therapy and the like, but preferably do not have the ability to express a protein or functional fragment of a protein. Among useful viruses or viral sequences which may be manipulated to provide the required RNA molecule to the mammalian cell *in vivo* are, without limitation, alphavirus, adenovirus, adeno-

15 associated virus, baculoviruses, delta virus, pox viruses, hepatitis viruses, herpes viruses, papova viruses (such as SV40), poliovirus, pseudorabies viruses, retroviruses, vaccinia viruses, positive and negative stranded RNA viruses, viroids, and virusoids, or portions thereof. These various viral delivery agents may be designed by applying conventional techniques such as described in M. Di Nocla *et al*, Cancer Gene Ther.,

20 5(6):350-6 (1998), among others, with the teachings of the present invention.

Another delivery agent for providing the information necessary for formation of the desired, above-described RNA molecules in the mammalian cell include live, attenuated or killed, inactivated donor cells which have been transfected or infected *in vitro* with a synthetic RNA molecule or a DNA delivery molecule or a delivery

25 recombinant virus as described above. These donor cells may then be administered to the mammal, as described in detail below, to stimulate the mechanism in the mammal which mediates this inhibitory effect. These donor cells are desirably mammalian cells, such as C127, 3T3, CHO, HeLa, human kidney 293, BHK cell lines, and COS-7 cells, and preferably are of the same mammalian species as the mammalian recipient. Such

30 donor cells can be made using techniques similar to those described in, e.g., Emerich

et al, *J. Neurosci.*, **16**: 5168-81 (1996). Even more preferred, the donor cells may be harvested from the specific mammal to be treated and made into donor cells by *ex vivo* manipulation, akin to adoptive transfer techniques, such as those described in D. B. Kohn *et al*, *Nature Med.*, **4**(7):775-80 (1998). Donor cells may also be from non-
5 mammalian species, if desired.

Finally, the composition of this invention can also include one or more of the selected agents which are described above. The composition can contain a mixture of synthetic RNA molecules described above, synthetic DNA delivery molecules described above, and any of the other delivery agents described above, such as
10 recombinant bacteria, cells, and viruses. The identity of the composition mixture may be readily selected by one of skill in the art.

B. Pharmaceutical (Therapeutic or Prophylactic) Compositions of the Invention

The compositions of this invention for pharmaceutical use desirably contain
15 the synthetic RNA molecule as described above or the agent which provides that RNA molecule to the mammalian cell *in vivo* in a pharmaceutically acceptable carrier, with additional optional components for pharmaceutical delivery. The specific formulation of the pharmaceutical composition depends upon the form of the agent delivering the RNA molecule.

20 Suitable pharmaceutically acceptable carriers facilitate administration of the polynucleotide compositions of this invention, but are physiologically inert and/or nonharmful. Carriers may be selected by one of skill in the art. Such carriers include but are not limited to, sterile saline, phosphate, buffered saline, dextrose, sterilized water, glycerol, ethanol, lactose, sucrose, calcium phosphate, gelatin, dextran, agar,
25 pectin, peanut oil, olive oil, sesame oil, and water and combinations thereof. Additionally, the carrier or diluent may include a time delay material, such as glycerol monostearate or glycerol distearate alone or with a wax. In addition, slow release polymer formulations can be used. The formulation should suit not only the form of

the delivery agent, but also the mode of administration. Selection of an appropriate carrier in accordance with the mode of administration is routinely performed by those skilled in the art.

Where the composition contains the synthetic RNA molecule or where the agent is another polynucleotide, such as, a DNA molecule, plasmid, viral vector, or recombinant virus, or multiple copies of the polynucleotide or different polynucleotides, etc., as described above, the composition may desirably be formulated as "naked" polynucleotide with only a carrier. Alternatively, such compositions desirably contain optional polynucleotide facilitating agents or "co-agents", such as a local anaesthetic, a peptide, a lipid including cationic lipids, a liposome or lipidic particle, a polycation such as polylysine, a branched, three-dimensional polycation such as a dendrimer, a carbohydrate, a cationic amphiphile, a detergent, a benzylammonium surfactant, or another compound that facilitates polynucleotide transfer to cells. Non-exclusive examples of such facilitating agents or co-agents useful in this invention are described in U. S. Patent Nos. 5,593,972; 5,703,055; 5,739,118; 5,837,533 and International Patent Application No. WO96/10038, published April 4, 1996; and International Patent Application No WO94/16737, published August 8, 1994, which are each incorporated herein by reference.

When the facilitating agent used is a local anesthetic, preferably bupivacaine, an amount of from about 0.1 weight percent to about 1.0 weight percent based on the total weight of the polynucleotide composition is preferred. See, also, International Patent Application No. PCT/US98/22841, which teaches the incorporation of benzylammonium surfactants as co-agents, administered in an amount of between about 0.001-0.03 weight %, the teaching of which is hereby incorporated by reference.

Where the delivery agent of the composition is other than a polynucleotide composition, e.g., is a transfected donor cell or a bacterium as described above, the composition may also contain other additional agents, such as those discussed in US Patents No. 5,824,538; 5,643,771; 5,877,159, incorporated herein by reference.

Still additional components that may be present in any of the compositions are, adjuvants, preservatives, chemical stabilizers, or other antigenic proteins. Typically, stabilizers, adjuvants, and preservatives are optimized to determine the best formulation for efficacy in the target human or animal. Suitable exemplary preservatives include chlorobutanol, potassium sorbate, sorbic acid, sulfur dioxide, propyl gallate, the parabens, ethyl vanillin, glycerin, phenol, and parachlorophenol. Suitable stabilizing ingredients which may be used include, for example, casamino acids, sucrose, gelatin, phenol red, N-Z amine, monopotassium diphosphate, lactose, lactalbumin hydrolysate, and dried milk. A conventional adjuvant is used to attract leukocytes or enhance an immune response. Such adjuvants include, among others, Ribi, mineral oil and water, aluminum hydroxide, Amphigen, Avridine, L121/squalene, D-lactide-polylactide/glycoside, pluronic ployois, muramyl dipeptide, killed *Bordetella*, and saponins, such as Quil A.

In addition, other agents which may function as transfecting agents and/or replicating agents and/or inflammatory agents and which may be co-administered with the composition of this invention, include growth factors, cytokines and lymphokines such as alpha-interferon, gamma-interferon, platelet derived growth factor (PDGF), colony stimulating factors, such as G-CSF, GM-CSF, tumor necrosis factor (TNF), epidermal growth factor (EGF), and the interleukins, such as IL-1, IL-2, IL-4, IL-6, IL-8, IL-10 and IL-12. Further, fibroblast growth factor, surface active agents such as immune-stimulating complexes (ISCOMS), Freund's incomplete adjuvant, LPS analog including monophosphoryl Lipid A (MPL), muramyl peptides, quinone analogs and vesicular complexes such as squalene and squalene, and hyaluronic acid may also be used administered in conjunction with the compositions of the invention.

The pharmaceutical compositions may also contain other additives suitable for the selected mode of administration of the composition. Thus, these compositions can contain additives suitable for administration via any conventional route of administration, including without limitation, parenteral administration, intraperitoneal administration, intravenous administration, intramuscular administration, subcutaneous administration, intradermal administration, oral administration, topical

administration, intranasal administration, intra-pulmonary administration, rectal administration, vaginal administration, and the like. All such routes are suitable for administration of these compositions, and may be selected depending on the agent used, patient and condition treated, and similar factors by an attending physician.

5 The composition of the invention may also involve lyophilized polynucleotides, which can be used with other pharmaceutically acceptable excipients for developing powder, liquid or suspension dosage forms, including those for intranasal or pulmonary applications. See, e.g., Remington: The Science and Practice of Pharmacy, Vol. 2, 19th edition (1995), e.g., Chapter 95 Aerosols; and International Patent
10 Application No. PCT/US99/05547, the teachings of which are hereby incorporated by reference. Routes of administration for these compositions may be combined, if desired, or adjusted.

15 In some preferred embodiments, the pharmaceutical compositions of the invention are prepared for administration to mammalian subjects in the form of, for example, liquids, powders, aerosols, tablets, capsules, enteric coated tablets or capsules, or suppositories.

20 The compositions of the present invention, when used as pharmaceutical compositions, can comprise about 1 ng to about 20 mgs of polynucleotide molecules as the delivery agent of the compositions, e.g., the synthetic RNA molecules or the delivery agents which can be DNA molecules, plasmids, viral vectors, recombinant viruses, and mixtures thereof. In some preferred embodiments, the compositions contain about 10 ng to about 10 mgs of polynucleotide sequences. In other embodiments, the pharmaceutical compositions contain about 0.1 to about 500 ngs

(7) a ss murine RNA polynucleotide sequence homologous to the top strand of the IL-12 p40 promoter,

(8) a ss murine RNA polynucleotide sequence homologous to the bottom strand of the IL-12 p40 promoter, and

5 (9) a ds RNA molecule comprised of murine RNA polynucleotide sequences homologous to the top and bottom strands of the IL-12 p40 promoter.

As a negative control the sense, anti-sense and ds RNAs derived from the HSV2 gD gene described in Example 1 are also used. Another control group is composed of mice receiving no RNA.

10 As described in Example 1, the various RNA molecules of (1)-(9) above are generated through T7 RNA polymerase transcription of PCR products bearing a T7 promoter at one end. In the instance where a sense RNA is desired, a T7 promoter is located at the 5' end of the forward PCR primer. In the instance where an antisense RNA is desired, the T7 promoter is located at the 5' end of the reverse 15 PCR primer. When dsRNA is desired both types of PCR products are included in the T7 transcription reaction. Alternatively, sense and anti-sense RNA are mixed together after transcription.

The PCR primers used in the construction of the RNA molecules of this Example are 5' to 3', with the top strand of the T7 promoter underlined.

20 Forward IL-12 genomic (hnRNA) [SEQ ID NO: 9]:

5' TCAGCAAGCACTTGCCAAACTCCTG 3' and Reverse IL-12 genomic (hnRNA) [SEQ ID NO: 10]: 5' GAGACAAGGTCTCTGGATGTTATTG 3';

T7 Forward IL-12 genomic (hnRNA) [SEQ ID NO: 11]:

5' GTAATACGACTCACTATAAGGGTCAGCAAGCACTTGCCAAACTCCTG 3'

25 and T7 Reverse IL-12 genomic (hnRNA) [SEQ ID NO: 12]:

5' GTAATACGACTCACTATAAGGGGAGACAAGGTCTCTGGATGTTATTG 3';

T7 Forward IL-12 promoter [SEQ ID NO: 13]:

5' GTAATACGACTCACTATAAGGGCCTATAAGCATAAGAGACGCCCTC 3' and Forward IL-12 promoter [SEQ ID NO: 14]:

30 5' CCTATAAGCATAAGAGACGCCCTC 3';

Reverse IL-12 promoter [SEQ ID NO: 15]:
5' GGCTGCTCCTGGTGCTTATATAC 3'
and T7 Reverse IL-12 promoter [SEQ ID NO: 16]:
5' GTAATACGACTCACTATAGGGGCTGCTCCTGGTGCTTATATAC 3';
5 T7 Forward IL-12 cDNA (mRNA) [SEQ ID NO: 17]:
5' GTAATACGACTCACTATAAGGGTGTGTCCCTCAGAAGCTAACCATC 3' and
Forward IL-12 cDNA (mRNA) [SEQ ID NO: 18]:
5' TGTGTCCTCAGAAGCTAACCATC 3';
Reverse IL-12 cDNA (mRNA) [SEQ ID NO: 19]:
10 5' GCAGGTGACATCCTCCTGGCAGGA 3'
and T7 Reverse IL-12 cDNA (mRNA) [SEQ ID NO: 20]:
5' GTAATACGACTCACTATAAGGGCAGGTGACATCCTCCTGGCAGGA 3'.
The genomic and PCR primer coordinates are based on the map
supplied in the following citation: Tone *et al*, *Eur. J. Immunol.*, **26**:1222-1227
15 (1996). The forward IL-12 genomic primer maps to coordinates 8301-8325. The
reverse IL-12 genomic primer maps to coordinates 8889-8913. The forward IL-12
promoter primer maps to coordinates 83-106. The reverse IL-12 promoter primer
maps to coordinates 659-682. The coordinates for the cDNA PCR primers is based on
GenBank Accession No. M86671. The forward IL-12 cDNA primer maps to
20 coordinates 36-58. The reverse IL-12 cDNA primer maps to coordinates 659-682.

B. Assay

Balb/c mice (5 mice/group) are injected intramuscularly or
intraperitoneally with the murine IL-12 p40 chain specific RNAs described above or
with controls identified above at doses ranging between 10 µg and 500 µg. Sera is
25 collected from the mice every four days for a period of three weeks and assayed for
IL-12 p40 chain levels using the Quantikine M-IL-12 p40 ELISA Assay (Genzyme).

According to the present invention, mice receiving ds RNA molecules
derived from both the IL-12 mRNA, IL-12 hnRNA and ds RNA derived from the IL-
12 promoter demonstrate a reduction or inhibition in IL-12 production. A modest, if

any, inhibitory effect is observed in sera of mice receiving the single stranded IL-12 derived RNA molecules, unless the RNA molecules have the capability of forming some level of double-strandedness. None of the HSV gD derived RNAs are expected to reduce or inhibit IL-12 *in vivo* in a specific manner..

5 **EXAMPLE 4: METHOD OF THE INVENTION IN THE PROPHYLAXIS OF DISEASE**

A. *In Vitro Assay*

10 Vero and/or BHK cells, seeded at a density of 20-30% confluency, are cultured in six-well plates at 37°C in DMEM with 10% FBS. When cells are 80-90% confluent, they are transfected with 2-3 µg of the HIV gag- and HSV gD- specific RNA molecules described in Example 1 using lipofectamine (Gibco-BRL) as a transfecting agent. The RNA molecules are also delivered in the absence of any known transfecting agent in amounts varying between 5 and 100 µg. Another group of cells receives no RNA.

15 Still other groups of Vero and/or BHK cells are similarly transfected with 2-3 µg of a double-stranded DNA plasmid, plasmid 24, which is described in U. S. Patent No. 5,851,804, incorporated herein by reference, which contains a sequence encoding the HSV2 gD protein under the control of the HCMV promoter and a SV40 polyA sequence.

20 The transfected cells are cultured at 37°C in DMEM with 10% FBS. At days 1, 2, 4 and 7 following transfection, cells are infected with HSV2 at a multiplicity of infection (MOI) of 0.1 in an inoculum of 250 µl DMEM. The inoculum is allowed to adsorb for 1 hour after which 2 mls of DMEM (10% FBS) is added per well. For those cells infected at 4 and 7 days post transfection, the cells are passaged 25 into a new six-well plate such that they are confluent at the time of infection. If the cells are not passaged, they become overcrowded.

At 36-48 hours post-infection, the cell lysates are assayed for viral titer by conventional plaque assay on Vero cells [Clinical Virology Manual, 2d edit., eds. S. Specter and G. Lancz, pp. 473-94 (1992)]. According to this invention, the cells

transfected with the ds DNA plasmid, APL-400-024, and with the ds RNA molecule containing a polynucleotide sequence of the gD2 antigen, cannot be productively infected with HSV2. All other cells are anticipated to become productively infected with HSV2.

5 *B. In Vivo Assay*

Using the HSV-gD specific RNA molecules described in Example 1, which do not have the ability to make HSVgD protein and HIV gag specific RNA molecules as controls, mice are evaluated for protection from HSV challenge through the use of the injected HSVgD specific RNA molecules of the invention.

10 Balb/c mice (5 mice/group) are immunized intramuscularly or intraperitoneally with the described RNA molecules at doses ranging between 10 and 500 µg RNA. At days 1, 2, 4 and 7 following RNA injection, the mice are challenged with HSV-2 (10^5 pfu in 30 µls) by intravaginal inoculation. Everyday post HSV-2 inoculation, the mice are observed for signs of infection and graded on a scale of 0-4.

15 Zero is no sign of infection; 1 denotes redness; 2 denotes vesicles and redness; 3 denotes vesicles, redness and incontinence; and 4 denotes paralysis.

According to the present invention, because the mice that receive dsRNA molecules of the present invention which contain the HSV gD sequence are shown to be protected against challenge. The mice receiving the HIV gag control RNA molecules are not protected. Mice receiving the ss RNA molecules which contain the HSV gD sequence are expected to be minimally, if at all, protected, unless these molecules have the ability to become at least partially double stranded *in vivo*. According to this invention, because the dsRNA molecules of the invention do not have the ability to make HSV gD protein, the protection provided by delivery of the RNA molecules to the animal is due to a non-immune mediated mechanism that is gene specific.

All above-noted published references are incorporated herein by reference. Numerous modifications and variations of the present invention are included in the above-identified specification and are expected to be obvious to one of skill in the art. Such modifications and alterations to the compositions and processes of the present 5 invention are believed to be encompassed in the scope of the claims appended hereto.

41. The composition according to claim 1, comprising a mixture of different said agents.

42. The composition according to claim 1 wherein said target polynucleotide sequence is a virus polynucleotide sequence necessary for replication and/or pathogenesis of said virus in an infected mammalian cell.

43. The composition according to claim 42, wherein said virus is selected from the group consisting of a DNA virus and a virus that has an intermediary DNA stage.

44. The composition according to claim 43, wherein said virus is selected from the group consisting of Retrovirus, Herpesvirus, Hepadenovirus, Poxvirus, Parvovirus, Papillomavirus, and Papovavirus.

45. The composition according to claim 44, wherein said virus is selected from the group consisting of HIV, HBV, HSV, CMV, HPV, HTLV and EBV.

46. The composition according to claim 1, wherein said target polynucleotide sequence is a tumor antigen or functional fragment thereof or a regulatory sequence of a virus-induced cancer, which antigen or sequence is required for the maintenance of said tumor in said mammal.

47. The composition according to claim 46, wherein said cancer is selected from the group consisting of HPV E6/E7 virus-induced cervical carcinoma, HTLV-induced cancer and EBV induced cancer.

48. The composition according to claim 1, wherein said target polynucleotide sequence is a polynucleotide sequence of an intracellular or extracellular pathogen necessary for replication and/or pathogenesis of said pathogen in an infected mammalian cell.

49. The composition according to claim 1 wherein said target polynucleotide sequence is a polynucleotide sequence of an abnormal cancer-causing sequence in a mammal which also possesses a normal copy of said sequence, and wherein the differences between the abnormal and the normal sequences are differences in polynucleotides.

50. The composition according to claim 49 wherein said abnormal sequence is a fusion of two normal genes.

51. The composition according to claim 50 wherein said target polynucleotide is the polynucleotide sequence spanning said fusion.

52. A pharmaceutical composition comprising a composition of any of claims 1-51, and an optional second agent that facilitates polynucleotide uptake in a cell, in a pharmaceutically acceptable carrier.

53. The composition according to claim 52, wherein said second agent is selected from the group consisting of a local anaesthetic, a peptide, a lipid including cationic lipids, a liposome or lipidic particle, a polycation, a branched, three-dimensional polycation, a carbohydrate, a cationic amphiphile, a detergent, a benzylammonium surfactant, or another compound that facilitates polynucleotide transfer to cells.

54. The composition according to claim 53 wherein said second agent is bupivacaine.

55. A method for treating a viral infection in a mammal, comprising:
administering to said mammal a composition according to claim 1, with
an optional second agent that facilitates polynucleotide uptake in a cell, in a
pharmaceutically acceptable carrier, wherein said target polynucleotide is a virus
polynucleotide sequence necessary for replication and/or pathogenesis of said virus in
an infected mammalian cell, in an amount effective to reduce or inhibit the function of
said viral sequence in the cells of said mammal.

56. A method for preventing a viral infection in a mammal, comprising:
administering to said mammal a composition according to claim 1, with
an optional second agent that facilitates polynucleotide uptake in a cell, in a
pharmaceutically acceptable carrier, wherein said target polynucleotide is a virus
polynucleotide sequence necessary for replication and/or pathogenesis of said virus in
an infected mammalian cell, in an amount effective to reduce or inhibit the function of
said viral sequence upon subsequent introduction of said virus into said mammalian
cells.

57. A method for treatment or prophylaxis of a virally induced cancer in a
mammal comprising:
administering to said mammal a composition according to claim 1, with
an optional second agent that facilitates polynucleotide uptake in a cell, in a
pharmaceutically acceptable carrier, wherein said target polynucleotide is a sequence
encoding a tumor antigen, a regulatory sequence, or a functional fragment thereof,
which antigen or sequence function is required for the maintenance of said tumor in
said mammal, in an amount effective to reduce or inhibit the function of said antigen in
said mammal.

58. A method for the treatment or prophylaxis of infection of a mammal by an intracellular or extracellular pathogen comprising administering to said mammal a composition according to claim 1, with an optional second agent that facilitates polynucleotide uptake in a pathogenic or mammalian cell, in a pharmaceutically acceptable carrier, wherein said target polynucleotide is a polynucleotide sequence of said pathogen necessary for replication and/or pathogenesis of said pathogen in an infected mammal or mammalian cell, in an amount effective to reduce or inhibit the function of said sequence in said mammal.

59. A method of treatment or prophylaxis of cancer in a mammal comprising administering to said mammal a composition according to claim 1, with an optional second agent that facilitates polynucleotide uptake in a cell, in a pharmaceutically acceptable carrier, wherein said target polynucleotide is a polynucleotide sequence of an abnormal cancer-causing sequence in a mammal which also possesses a normal copy of said sequence, and wherein the differences between the abnormal sequence and said normal sequence are differences in polynucleotides, in an amount effective to reduce or inhibit the function of said abnormal sequence in said mammal.

60. A method for treating a disease or disorder in a mammal comprising: administering to said mammal having a disease or disorder characterized by expression of polynucleotide product not found in a healthy mammal, a composition according to claim 1, wherein said target polynucleotide sequence is a polynucleotide sequence which expresses said polynucleotide product or regulatory sequence necessary to expression of said product, in an amount effective to reduce or inhibit the function of said target polynucleotide product in the cells of said mammal.

61. Use of a composition according to claim 1, with an optional second agent that facilitates polynucleotide uptake in a cell, in a pharmaceutically acceptable carrier, wherein said target polynucleotide is a virus polynucleotide sequence necessary for replication and/or pathogenesis of said virus in an infected mammalian cell, in the preparation of a medicament for treating a viral infection in a mammal.

62. Use according to claim 61, wherein said composition is in an amount effective to reduce or inhibit the function of said viral sequence in the cells of said mammal.

63. Use according to claim 61, wherein said composition is in an amount effective to reduce or inhibit the function of said viral sequence upon subsequent introduction of said virus into said mammalian cells.

64. Use of a composition according to claim 1, with an optional second agent that facilitates polynucleotide uptake in a cell, in a pharmaceutically acceptable

<223> Description of Artificial Sequence: reverse IL-12
genomic

<400> 10
gagacaaggctctggatgttattg

25

<210> 11
<211> 46
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: T7 forward
IL-12 genomic

<400> 11
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46

<210> 12
<211> 46
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: T7 reverse
IL-12 genomic

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46

<210> 13
<211> 45
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: T7 forward
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45

<210> 14
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<212> DNA
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<220>
<223> Description of Artificial Sequence: forward IL-12 promoter

<400> 14
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24

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<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: reverse IL-12 promoter

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23

<210> 16
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<220>
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<212> DNA
<213> Artificial Sequence

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<223> Description of Artificial Sequence: T7 forward IL-12 cDNA

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cDNA

<400> 19
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24

<210> 20
<211> 45
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: T7 reverse
IL-12 cDNA

<400> 20
gtaatacgac tcactatagg ggcaggtgac atcctcctgg cagga

45

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(71) Applicant (for all designated States except US): AMERICAN HOME PRODUCTS CORPORATION [US/US]; Five Giralta Farms, Madison, NJ 07940 (US).

(72) Inventors; and

(75) Inventors/Applicants (for US only): PACHUK, Catherine [US/US]; 3212 New Park Circle, Lansdale, PA 19446 (US). SATISHCHANDRAN, C. [US/US]; 605 Shepard Drive, Lansdale, PA 19446 (US).

(74) Agents: BAK, Mary, E. et al.; Howson and Howson, Spring House Corporate Center, P.O. Box 457, Spring House, PA 19477 (US).

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(54) Title: METHODS AND COMPOSITIONS FOR INHIBITING THE FUNCTION OF POLYNUCLEOTIDE SEQUENCES

(57) Abstract: A therapeutic composition for inhibiting the function of a target polynucleotide sequence in a mammalian cell includes an agent that provides to a mammalian cell an at least partially double-stranded RNA molecule comprising a polynucleotide sequence of at least about 200 nucleotides in length, said polynucleotide sequence being substantially homologous to a target polynucleotide sequence. This RNA molecule desirably does not produce a functional protein. The agents useful in the composition can be RNA molecules made by enzymatic synthetic methods or chemical synthetic methods *in vitro*; or made in recombinant cultures of microorganisms and isolated therefrom, or alternatively, can be capable of generating the desired RNA molecule *in vivo* after delivery to the mammalian cell. In methods of treatment of prophylaxis of virus infections, other pathogenic infections or certain cancers, these compositions are administered in amounts effective to reduce or inhibit the function of the target polynucleotide sequence, which can be of pathogenic origin or produced in response to a tumor or other cancer, among other sources.

INTERNATIONAL SEARCH REPORT

Interr:	1al Application No
PCT/US 00/10555	

A. CLASSIFICATION OF SUBJECT MATTER					
IPC 7	C12N15/11	C12N15/63	C12N15/70	C12N15/86	C07K14/01
	C07K14/08	A61K48/00	A61K38/16	A61P35/00	
According to International Patent Classification (IPC) or to both national classification and IPC					
B. FIELDS SEARCHED					
Minimum documentation searched (classification system followed by classification symbols)					
IPC 7 C12N A61K					
Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched					
Electronic data base consulted during the international search (name of data base and, where practical, search terms used)					
EPO-Internal, BIOSIS					
C. DOCUMENTS CONSIDERED TO BE RELEVANT					
Category	Citation of document, with indication, where appropriate, of the relevant passages				Relevant to claim No.
X	<p>MONTGOMERY M K ET AL: "Double-stranded RNA as a mediator in sequence-specific genetic silencing and co-suppression" TRENDS IN GENETICS, NL, ELSEVIER SCIENCE PUBLISHERS B.V. AMSTERDAM, vol. 14, no. 7, 1 July 1998 (1998-07-01), pages 255-258, XP004124680 ISSN: 0168-9525 the whole document</p>				1